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TITLE: Co-Operation Between FADD and Bin1 in Prostate Cancer Apoptosis

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Introduction.

Evasion of apoptosis is a hallmark of cancer (Hanahan and Weinberg, 2000). Consequently, it is sometimes thought that cancer cells are generally resistant to apoptosis while normal cells are sensitive. In fact cancer cells are actually closer to their apoptotic threshold than their normal counterparts and therefore often undergo apoptosis more easily in response to diverse apoptotic stimuli (Evan and Vousden, 2001). This apoptosis sensitization occurs because growth promoting oncogenic events such as Myc expression raise the levels of caspases and other apoptotic proteins or make it easier to activate these molecules and thus reduce the threshold at which apoptosis is activated. However, it is not clear if this is the only apoptotic barrier that cancer cells must overcome as they become transformed. Are there also specific apoptosis pathways that inhibit cancer development and are active in normal cells and specifically inactivated during tumor development? We hypothesized that such a pathway would have the unusual characteristic of working in normal cells but not in cancer cells. Furthermore unlike most apoptotic stimuli, which usually work better in cancer cells than their normal counterparts because they are closer to their apoptotic threshold, signaling proteins and physiological stimuli that activate this kind of pathway should induce specific apoptosis responses in normal cells that should be selectively inhibited in cancer cells without affecting responsiveness to other apoptotic stimuli. The apoptosis pathway we study that is induced by FADD-DD has these characteristics because it works in normal epithelial cells but does not work in immortalized epithelial cells. Tumor cells are not normally sensitive to FADD-DD-induced apoptosis, however, we discovered that a prostate cell line (LNCaP) could become sensitive if we express the Bin1 tumor suppressor. This project is designed to further investigate this response.

Body.

Our goals for this funding period were to further determine if apoptosis induced by FADD-DD plus Bin1 in LNCaP tumor cells has similar characteristics to that induced by FADD-DD alone in normal prostate cells. Such characteristics include killing might involve both caspases and a serine protease. We then proposed to identify regions of the Bin1 protein that are responsible for this effect.

We were held up in our studies towards task 1 because of technical difficulties with our time-lapse microscope that meant we were unable to perform our planned studies on membrane blebbing etc. (tasks 1b and 1d). The technical problems with the equipment are now resolved and we will be able to return to these studies in the next funding period. We have made progress towards tasks 1a and have been able to begin our studies towards 1d as outlined below. Because of these problems, we focused our efforts on task 2 and task 4, which were originally planned for years 2 and 3. While these changes alter the timing of the experiments as proposed in the original SOW, they do not alter our overall objectives because none of our experiments relied upon results from previous experiments.

Task 1 a. (originally intended for months 1-3). Determine if zVAD and AEBSF affect LNCaP apoptosis in response to FADD-DD and Bin1.

We previously showed that zVAD and AEBSF (i.e. caspase and serine protease inhibitors) must be combined to prevent FADD-DD induced death of normal prostate epithelial cells (Thorburn et al., 2003). We performed injection experiments to test if this was also true in LNCap cells expressing both Bin1 and FADD-DD. Figure 1 shows that neither zVAD or AEBSF on their own are able to inhibit cell death however the combination does effectively inhibit LNCaP death in response to expression of both FADD-DD and Bin1.

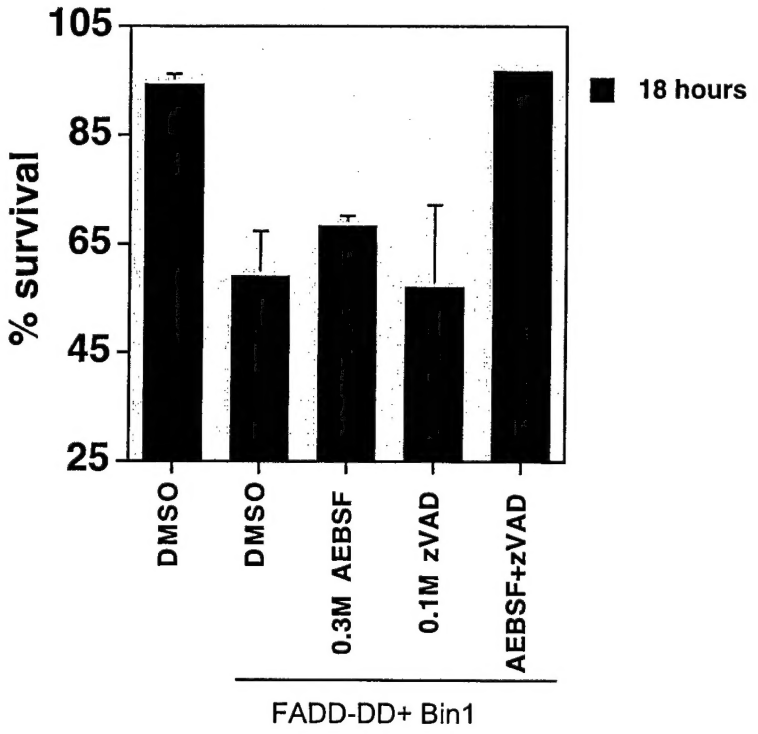

Fig. 1. LNCaP cell death in response to FADD-DD can only be inhibited by combining zVAD and AEBSF. LNCaP cells were injected with YFP control (first bar) or FADD-DD plus Bin1 (all other bars) then treated with the serine protease inhibitor AEBSF or the caspase inhibitor zVAD as described. Apoptosis was assayed as in our previous studies. Note that as before Bin1 plus FADD-DD resulted in apoptosis, this was not inhibited by either inhibitor alone but the combination of inhibitors resulted in equivalent survival to that observed with YFP injection in untreated cells. These data indicate that like FADD-DD-induced death in normal cells both caspases and serine proteases are involved in cell death when Bin1 and FADd-DD are combined in LNCaP cells.

Task 1d (originally intended for months 6-9). Perform western blotting experiments to assess caspase activation. The above data suggest that caspases are activated. We therefore planned to construct adenoviruses to express Bin1 and FADD-DD then perform western analysis. We have not yet completed these studies but have been able to show that caspases are activated as demonstrated by cleavage of the hallmark caspase substrate PARP (Fig. 2).

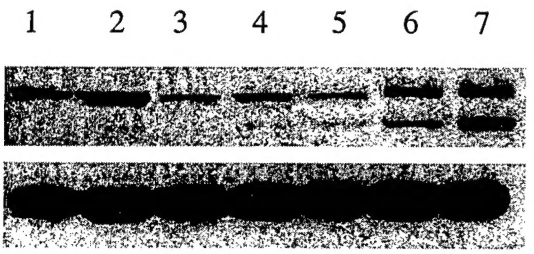

Fig. 2. Caspase activation by Bin1+ FADD-DD in LNCaP cells. LNCaP cells were infected with adenoviruses expressing FADD-DD and Bin1 then assessed for PARP cleavage by western blotting. Lane 1 shows uninfected cells, which have intact PARP, Lane 2 shows cells infected with control YFP virus, lane 3 shows cells infected with Bin1 virus, lane 4 shows cells infected with FADD-DD virus, lane 5 shows cells infected with a FADD-DD point mutant that cannot kill normal cells (unpublished data) plus Bin1, lane 6 shows cells infected with FADD-DD plus Bin1 lane 7 shows uninfected cells treated with sorbitol to induce apoptosis as a positive control. Note that only the combination of FADD-DD plus Bin1 virus leads to significant PARP cleavage. These data indicate that caspases are activated in LNCaP cells by the combination of FADD-DD plus Bin1.

Task 2. (originally intended for months 9-18). Identify regions of Bin1 that co-operate with FADD-DD.

Bin1 and FADD-DD were each unable to kill LNCaP cells when expressed on their own. In contrast, as shown in our original grant application, co-expression of FADD-DD and Bin1 resulted in efficient cell death (Fig. 3). This suggests that Bin1 is involved in the FADD-DD pathway. Co-expression of FADD-DD and a Bin1 mutant (Bin1ΔBAR) that deletes the conserved amino acids 143-148 that define the BAR domain, did not result in cell death. Point mutations of the two conserved Aspartic acid residues in the BAR domain (Bin1DD-AA) or a conserved Leucine (Bin1L-E) also prevented cooperation with FADD-DD. In contrast, a Bin1 mutant that lacks the Myc-binding domain (Bin1ΔMyc) co-operated with FADD-DD as well as the wildtype protein. These data indicate that cooperation between FADD-DD and Bin1 requires specific activities of Bin1 including a functional BAR domain but that Myc binding is not involved in the response and achieve the goals outlined in tasks 2a-c.

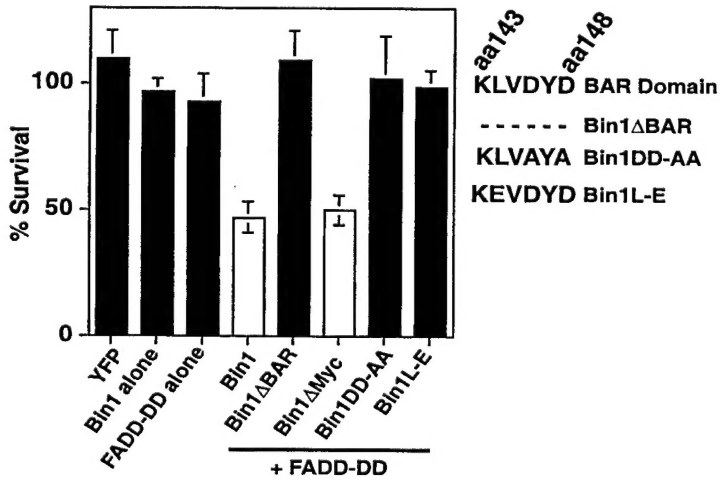

Fig. 3. Bin1 makes prostate tumor cells susceptible to FADD-DD-induced apoptosis. LNCaP cells were injected with the indicated plasmids and cell survival was determined. Bin1 or FADD-DD alone did not kill. Co-expression of FADD-DD with wildtype Bin1 (white bar) resulted in cell death. Co-expression of FADD-DD with Bin1DBAR lacking six conserved amino acids from the BAR domain or mutations of the conserved Aspartic acids (Bin1DD-AA) or Leucine (Bin1L-E) did not result in cell death. A Bin1 mutant that lacks the Myc binding domain (Bin1DMyc) co-operated with FADD-DD (white bar).

We next tested other point mutants in the BAR domain. Fig. 4 shows that all the mutants tested so far abolished the co-operation with FADD-DD. Together, these data show that the BAR domain is essential for co-operation with Bin1.

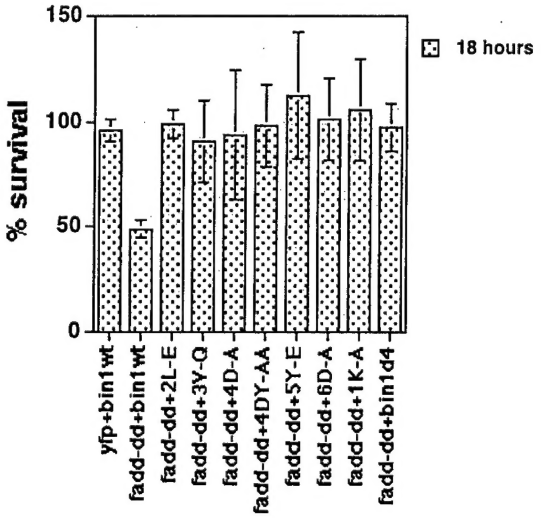

Fig. 4. BAR domain point mutants fail to co-operate with FADD-DD. Wildtype Bin1 or various double point mutants in the conserved six amino acid sequence shown in Fig 3 above were tested for their ability to co-operate with FADD-DD to induce LNCaP cell death. None of the mutants could co-operate indicating that a functional BAR domain is essential for co-operation.

Task 4 (originally intended for months 24-36).

In this part of our work, we proposed to inhibit Bin1 in normal epithelial cells and determine if this blocked FADD-DD-induced apoptosis. We proposed to do this using siRNA , shRNA and antisense approaches. We now have an alternate strategy that will provide a more definitive approach to this problem. We recently showed that primary mouse epithelial cells have the same response to FADD-DD as human cells, i.e. they undergo apoptosis while immortalized cells do not. We have performed these studies in mouse breast cells and mouse prostate cells with identical results (in a different project, DAMD17-02-1-0612, that addresses another aspect of the FADD pathway we are now using this approach in breast cells). Fig. 4 shows data from prostate cells.

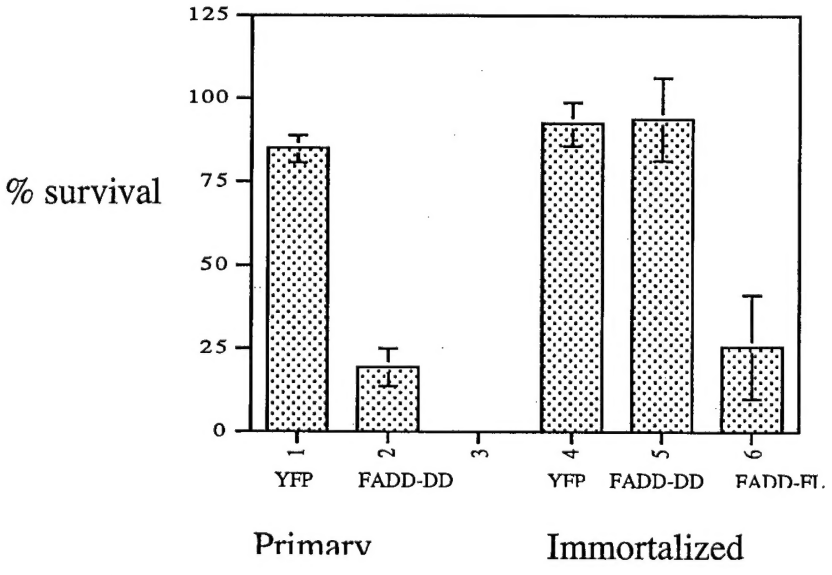

Fig. 4. Mouse prostate epithelial cells undergo apoptosis in response to FADD-DD. Primary and spontaneously immortalized mouse prostate epithelial cells were isolated and injected with YFP control of FADD-DD or a full-length FADD molecule (FADD-FL) that unlike FADD-DD can activate caspase-8 (this serves as a control for specificity of the apoptosis resistance). FADD-DD killed primary cells but not immortalized cells, this immortalization resistance was specific to the FADD-DD pathway because a FADD molecule that can activate caspase-8 is not affected.

This result is important because it means that we can now use the power of mouse genetic approaches to test if Bin1 is required for this effect. Bin1 knockout animals die shortly after birth (Muller et al., 2003) and we therefore cannot isolate prostate epithelial cells from these animals. We have therefore extended our collaboration as described in the original grant application with Dr. Prendergast to obtain access to his "floxed" Bin1 animals. These animals have loxP sites flanking their Bin1 gene that allows knockout of the gene by Cre recombinase. We have cultured prostate epithelial cells from these animals and are now ready to knockout the gene using a Cre adenovirus. This will allow a better way to answer the questions posed in task 4 of the SOW because it will allow us to work with prostate cells that contain no Bin1 and ask if FADD-DD can induce apoptosis.

Key research accomplishments.

- We showed that LNCap cell death induced by the combination of FADD-DD and Bin1 can be inhibited only when caspases and serine proteases are inhibited. Neither class of inhibitor on its own prevented death.
- We showed that caspase activity is induced by Bin1 plus FADD-DD in LNCaP cells. Caspase activity is not induced by Bin1 alone, FADD-DD alone or Bin1 plus a FADD-DD point mutant that is inactive.
- We identified the Bin1 BAR domain as being required for co-operation with FADD-DD and showed that point mutants in this domain abolish co-operation. These data achieve the goals set out in task 2, which was originally intended for months 9-18.
- We showed that mouse prostate cells behave like human prostate cells and undergo FADD-DD induced apoptosis that is inhibited in immortal cells. This allows us to use the powerful approaches afforded by gene knockouts to study this pathway. In this respect, we have cultured primary mouse prostate epithelial cells from animals with floxed Bin1 gene s and are currently knocking out the gene with a Cre adenovirus. This provides a more rigorous approach to achieve the goals in task 4, which were originally intended for year 3 of the grant.

Reportable outcomes.

None. We hope to submit a manuscript for publication describing these data within the next funding period of this grant.

Conclusions.

We completed task 1a and showed that Bin1 and FADD-DD have the same requirements for killing LNCaP cells as FADD-DD does for killing normal prostate cells. Technical problems with our microscopy equipment caused delays in our experiments towards tasks 1b and c (originally proposed for months 3-9), and we have not fully completed our studies for task 1d although our current experiments (see Fig. 2) show that caspases are indeed activated. Because of these difficulties, we instead completed task 2, which was originally planned to be completed in year 2 and have developed a better approach to deal with task 4 (originally intended for year 3). Together our data show that Bin1 co-operates with FADD-DD to induce LNCaP cell death, that this death involves both caspases and serine proteases and is therefore like that observed by our group previously

in normal cells expressing FADD-DD on its own. We have shown that this response requires the Bin1 BAR domain but does not involve the other major characterized functional domain in the protein (the Myc binding domain) or any of the other putative domains in Bin1.

What does this mean for prostate cancer?

Because the FADD-DD pathway is selectively inactivated at an early step in prostate (and also breast) cancer development, it may represent an early link between cell growth disregulation and apoptosis regulation that is important for cancer development. The studies supported by this grant show that this pathway involves the tumor suppressor Bin1 and thus provide new insights into why Bin1 loss, which is known to occur in prostate cancer, promotes development of the disease. Further understanding of these mechanism as outlined in the remaining parts of our study in the SOW and from other related projects may therefore identify new therapeutic targets for manipulating this pathway. If we can find ways to do this, we may be able to re-activate this apoptotic pathway in prostate cancer cells, which should result in tumor cell-specific cell killing that could be a useful treatment to limit the development of the disease.

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